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Myroides odoratimimus, a biocontrol agent from the rhizosphere of tobacco with potential to control *Alternaria alternata*

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Abstract Bacteria from the tobacco rhizosphere were isolated and evaluated for their potential of producing volatile compounds against tobacco brown spot pathogen *Alternaria alternata*. Among the 82 strains of bacteria isolated from tobacco rhizosphere, 22 produced volatiles against the fungal pathogen *A. alternata* and showed an inhibition rate of over 50 % on *A. alternata.* The inhibition rate of strain T45 was the most pronounced, reaching 78.89 %. Sequence analysis of 16S rDNA of T45 identified it as *Myroides odoratimimus*. Scanning electron microscopy revealed that the volatile compounds it produced strongly suppressed mycelial growth, conidial germination, and sporulation, leading to mycelial fragmentation, surface crumpling, and damage to hyphae morphology. A sealed plate experiment showed volatile compounds generated from *M. odoratimimus* T45 exhibited inhibitory effect on the growth of eight plant pathogenic fungi (10.52–88.20 %), the inhibition was most pronounced on *Valsa mali* while the least pronounced inhibitory effect was on *Colletotrichum orbiculare*. Headspace solid-phase microextraction gas chromatography mass spectrometry analysis revealed a total of 24 compounds in the volatiles produced by *M. odoratimimus*. The main components of volatiles primarily included alcohols, aldehydes, acids, ketones, esters, and hydrocarbons. At a concentration of 100 µl per dish, isoamyl alcohol, isobutyric acid and isovaleric acid were able to completely inhibit the mycelial growth of *A. alternata*. This is the first report on using volatiles produced by *M. odoratimimus* T45 as biocontrol agents against *A. alternata*. The studies indicated *M. odoratimimus* T45 and volatile compounds isoamyl alcohol, isobutyric acid and isovaleric acid it produced could be potential biocontrol agents suitable for use against *A. alternata*.

Keywords *Myroides odoratimimus* · Volatile compounds · Antifungal activity · *Alternaria alternata*

Introduction

Alternaria alternata is an important type of plant pathogen. It has a wide range of hosts to which it can cause serious damage. It causes tobacco brown spot, which is a major leaf disease that emerges during the late stage of tobacco maturity. It has been observed in several tobacco-growing parts in China, where it directly affects the yield and quality of tobacco. Currently, chemical-control-based methods are the most commonly used in China for prevention and

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control of tobacco brown spot. However, use of chemical fungicides may lead to environmental pollution caused by pesticide residues, pathogen fungicide-resistance, and serious fungicide-induced damage to tobacco (Yi and Xiao 2003). Biocontrol, due to its environmental friendliness, is considered a good alternative to chemical fungicides for control of plant diseases, so screening and utilization of biocontrol resources are of considerable significance in the biocontrol of plant diseases.

Volatile products of microbial metabolism can often prevent fungal growth, prevent disease, promote growth, or induce resistance. These are an important class of biocontrol resources. McCain (1966) reported the inhibitory effect of *Streptomyces griseus* on the spore formation of *Gloeosporium aridum* (Li et al. 2012a). Chen et al. (2008) found that the volatile products of *Bacillus subtilis* could inhibit the spore germination and germination tube elongation of *Botrytis cinerea*. Koitabashi (2005) reported that the volatiles isolated from the metabolites of *Irpex lacteus* controlled the powdery mildew of greenhouse parsley caused by *Oidium* sp. satisfactorily. Zhang et al. (2008) showed that volatiles produced by *B. subtilis* GB03 could promote *Arabidopsis* growth by enhancing its photosynthesis. Volatile antimicrobial substances have several properties not shared by non-volatile antimicrobial substances, including deep penetration and even distribution. This tends to render their disease-preventive effects more pronounced and broadens their range of application (Fernando et al. 2005). Studies have shown that many microbes, including fungi and bacteria, can produce volatile antimicrobial substances (Li et al. 2010; Wenke et al. 2012; Zhou et al. 2011). Current studies on the biocontrol of *A. alternata* have focused on microbes that produce non-volatile antifungal substances, on *Trichoderma* (Fang et al. 2005), *Actinomyces* (Zhan et al. 2008), parasitic bacteria (Li et al. 1996), and antagonistic bacteria (Zhang et al. 2005). There have been relatively few investigations of microbes that produce volatile antifungal substances that inhibit *A. alternata* and relevant analysis of volatiles. This study was conducted to screen antifungal volatile producing-bacteria for biocontrol of *A. alternata* and to investigate the underlying action mode and to identify and analyze the antifungal volatile compounds.

Materials and methods

Test pathogen and bacterial strains

Alternaria alternata and eight common plant pathogenic fungi (*B. cinerea, Botryosphaeria dothidea*, *Valsa mali*, *Colletotrichum capsici*, *Colletotrichum orbiculare*, *Glomerella cingulata*, *Fusarium oxysporum* f. sp. *lycopersici*, and *F. oxysporum* f. sp. *vasinfectum*) were all provided by the Key Laboratory of Tobacco Pest Monitoring and Integrated Management, Tobacco Research Institute of Chinese Academy of Agricultural Sciences, China. Prior to the experiment, mycelial plugs at the edge of the pathogen stored in the refrigerator were placed on a fresh potato dextrose agar medium (PDA) at 26 °C for seven days.

The soil dilution plate method was used (Haddad et al. 2009), and 82 strains of bacteria were isolated from tobacco rhizospheric soil collected from Hubei Province, China. These were numbered T1–T82, and stored at −20 °C in glycerol. Before use, freshly thawed bacteria were placed in LB broth medium at 28 °C for 24 h. Single colonies were obtained by streaking. Before the sealed plate experiment, a single colony was picked in LB at 28 °C and subjected to shaking culture at 150 rpm for 24 h.

Screening of isolates against *A. alternata*

The sealed-plate method was used to assess the inhibitory effects of the 82 bacterial strains on *A. alternata* (Wan et al. 2008). Briefly, a hole puncher 5 mm in diameter was used to produce a mycelial plug from the periphery of a medium covered with *A. alternata*. The mycelial plug was then seeded on a new PDA plate for culturing at 26 °C for 12 h so that it adhered to the medium. After 24 h of culture, 100 µl bacterial broth was aspirated and evenly applied to an LB plate. A PDA plate containing *A. alternata* was inverted and placed on top of the LB plate. The two plates were then sealed with Parafilm plastic wrap. In the control group, the LB plate was not coated with bacteria. The sealed plates representing all groups were sealed in plastic bags and incubated at 26 °C for seven days. The criss-cross method was used to determine the diameters of the *A. alternata* colonies in the different groups, and the growth inhibition rate of the corresponding bacterium was calculated by the percentage reduction in diameter of treated colonies

compared to the control. Each treatment group contained five replicates. The bacterial strain with the highest inhibition rate was selected and studied further.

Identification of antagonistic bacteria

16S rDNA sequencing and phylogenetic analysis were used for molecular identification for the bacterial strains that had high inhibition rates. A bacterial genome extraction kit (Sangon Biotech) was used to extract bacterial genomic DNA. Using genomic DNA as a template and primers 27F: (5′-AGAGTTTGATC CTGGCTCAG-3′) and 1492R: (5′-GGTTACCTTGT-TACGACTT-3′), 16S rDNA was amplified from the bacterial strain. The PCR products obtained were purified via gel extraction (TransGen Biotech) and then sent to Sangon Biotech Co., Ltd., China for sequencing. The sequence was submitted to GenBank database for homology analysis by using the BLAST program and was also compared to the type strain sequence of the closest relative (http://www.straininfo.net/). Clustal 1.83 was used for multiple sequence alignment. The neighbor-joining method in Mega4.0 was used to construct a phylogenetic tree.

Inhibitory effect of volatiles from antagonistic bacteria on *A. alternata*

Inhibitory effect on mycelial growth

The bacterial strain with the highest rate of inhibition was subjected to the sealed plate method with *A. alternata* as described in the "Screening of isolates against *A. alternata*" section. The pathogen colony was cut from the edge of the medium plate, fixed in 3 % glutaraldehyde for 4 h, rinsed with 0.1 M phosphate buffer, fixed in 1 % osmic acid for 90 min, and rinsed with 0.1 M phosphate buffer. Next, it was subjected to serial dehydration using ethanol at concentrations of 30, 50, 70, 80, and 100 %, and twice in 100 % ethanol. Each dehydration process lasted 10 min. Then it was subjected to isoamyl acetate replacement, once in 50 %, and twice in 100 % isoamyl acetate. An XD-1 carbon dioxide critical point dryer (Eiko, Japan) was using for drying, and an IB-3 ion sputter coater (Eiko, Japan) was used for spray gold plating. A JSM-840 scanning electron microscope (Jeol, Japan) was used for observation. A sterile inoculating needle was used to pick mycelia on the edge of the medium and uniformly applied on the sterile water in the middle of a sterile glass slide, and the morphology of hyphae was observed under an optical microscope. For each treatment group, there were three replicates and at least five fields of view were observed for each replicate.

Inhibitory effect on conidial germination (Li et al. 2012b)

Alternaria alternata was inoculated on PDA medium for culturing at 26 °C for seven days, rinsed with sterile water, and then subjected to centrifugation at 3,000 r min^{-1} for 3 min to remove the mycelium. The collected supernatant, i.e., the conidium suspension, was diluted to concentration 10^2 conidia ml^{-1}. Then 100 µl conidium suspension was aspirated and evenly applied to a PDA plate, and the culture of the selected bacterium was applied to an LB plate. The sealed plate method was used as described in the "Screening of isolates against *A. alternata*" section. The group in which the LB plate was not inoculated with bacteria served as the control. Each treatment group contained three replicates and the experiment was repeated three times.

Antifungal activity of volatiles from antagonistic bacteria against eight plant pathogenic fungi

The effect of volatile compounds from antagonistic bacteria on mycelial growth of eight plant pathogenic fungi (*B. cinerea*, *B. dothidea*, *V. mali*, *C. capsici*, *C. orbiculare*, *G. cingulata*, *F. oxysporum* f. sp. *lycopersici*, *F. oxysporum* f. sp. *vasinfectum*) was evaluated using the sealed-plate method as described in the "Screening of isolates against *A. alternata*" section. The group in which the LB plate was not inoculated with bacteria served as the control and the experiment was repeated three times. The inhibition rate of mycelial growth was calculated as described in the "Screening of isolates against *A. alternata*" section.

GS/MS analysis of volatile antifungal compounds

Headspace solid-phase microextraction gas chromatography mass spectrometry (HS-SPME-GC/MS) was used to identify the volatile compounds produced by

the inhibitory bacterium. In a 15 ml headspace vial, 9 ml bacterial culture, and 0.1 g sodium sulfate were added, and a magnetic stirrer was placed in the vial, which was then secured in a constant temperature water bath. Then 75 μm Carboxen®/Polydimethylsiloxane (CAR/PDMS) fiber was used to penetrate the vial stopper, and the piston was pressed so that the fiber end protruded from the stainless steel tube and was exposed to the air above the sample. At 50 °C, the sample was subjected to magnetic stirring for 1 h. Volatile compounds of LB broth served as the control. After extraction, the ends of the fiber were quickly retracted and the head of the needle head was pulled out. The vial was then immediately inserted into the gasification chamber of the GC analyzer. The fiber was pushed out for desorption at 250 °C for 2 min. GC/MS conditions were as described in a previous study (Xu et al. 2004). The NIST 08 Mass Spectral Library was searched for identification, and compounds with ≥80 % similarity were identified as the same compounds.

Antifungal assay of identified volatile compounds

Pure commercial compounds (technical grade) were used to study the antifungal effects of select individual volatile compounds, and the two-compartment agar-plate experiment was conducted (Gu et al. 2007). Briefly, in a two-compartment plastic plate, PDA medium was poured into one compartment, and a plug of *A. alternata* plug 5 mm in diameter was inoculated into the medium. 100 μl pure commercial compound was added to the other compartment (Fernando et al. 2005). Sterile distilled water (SDW) served as a control. The plate was sealed with plastic wrap and placed at 26 °C for five days. The criss-cross method was used to calculate the rate of inhibition of growth. Each treatment group contained five replicates.

Data analyses

The data were subjected to analyses of variance (ANOVA) using SPSS 18.0 Windows Software (SPSS Inc., Chicago, USA). For percentage data, an arcsine transformation was applied prior to ANOVA. Means were separated by Fisher's Protected least significant difference (LSD) test at the level $P = 0.05$.

Results

Screening of bacteria producing antifungal volatiles

A total of 82 strains of bacteria isolated from tobacco rhizosphere collected from Hubei Province were found to produce volatile substances that inhibited *A. alternata* to varying degrees using the sealed plate analysis (Fig. 1). Among these, 50 strains showed an inhibition rate over 30 %, and 22 showed an inhibition rate over 50 % (Table 1). Four isolates inhibited *A. alternata* by more than 70 %: T47 (71.19 %), T65 (72.83 %), T79 (76.56 %), and T45 (78.89 %). T45, which inhibited the growth of *A. alternata* most prominently, was selected for further analysis.

16S rDNA identification of strain T45

The bacterial genomic DNA was extracted, amplified, and then subjected to sequencing. The 16S rDNA sequence obtained in this way was searched in GenBank database for homology. The sequence shared 99 % similarity to that of *Myroides odoratimimus*. A phylogenetic tree (Fig. 2) was constructed. Based on its taxonomic status, the sample was identified as *M. odoratimimus* with Genbank accession number KF758445.

Inhibitory effect of volatiles on *A. alternata*

Figure 3 shows the inhibitory effect of bacterial *M. odoratimimus* T45 on *A. alternata*. After seven days of culture, the *A. alternata* in the control group covered 2/3 of the dish (Fig. 3a). In the PDA plate

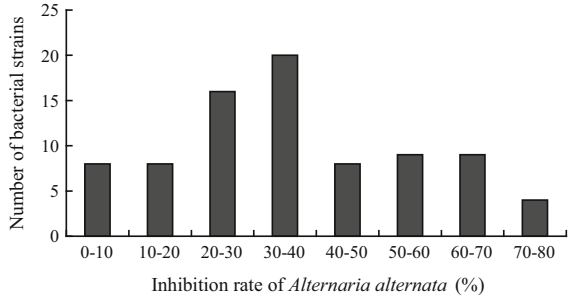

Fig. 1 Distribution of inhibition rates of strains isolated from tobacco rhizospheric soil against mycelial growth of *A. alternata*

Table 1 Inhibition rate of volatiles from rhizobacteria against mycelial growth of *A. alternata*

Code name of isolates	Inhibition rate[1] (%)
T45	78.89 ± 5.22^{a}
T79	76.56 ± 7.89^{ab}
T65	72.83 ± 10.43^{abc}
T47	71.19 ± 11.27^{bcd}
T24	68.07 ± 6.15^{cde}
T52	67.69 ± 4.77^{cde}
T46	67.36 ± 10.30^{cdef}
T39	66.33 ± 10.21^{cdefg}
T64	64.37 ± 5.66^{defgh}
T20	64.34 ± 11.72^{defgh}
T13	62.08 ± 3.76^{efghi}
T4	61.76 ± 2.50^{efghij}
T63	60.13 ± 0.62^{fghij}
T69	59.21 ± 4.97^{ghij}
T78	58.98 ± 7.26^{hij}
T40	55.89 ± 9.66^{ijk}
T33	55.20 ± 2.90^{ijk}
T44	54.55 ± 9.17^{ik}
T55	50.68 ± 3.31^{k}
T54	50.46 ± 3.52^{k}
T68	50.32 ± 4.29^{k}
T42	50.11 ± 4.52^{k}

Values are expressed as means of five replicates ±SD, inhibition percentage against *A. alternata* ($F = 7.19$, $df = 21, 88$, $P < 0.05$). Values within a column followed by the same lower case letters are not significantly different ($P < 0.05$)

[1] Inhibition rate was calculated as percentage mycelial growth inhibition

cultured with an inversed LB plate containing T45, the growth *A. alternata* was slow, and the colonies were yellowish white in color, demonstrating a clear inhibitory effect (Fig. 3b). Mycelia were picked from the edge of the pathogen plate in the control group and the treatment group, and were examined under an optical microscope. In the control group, *A. alternata* produced a large number of conidia, and the mycelia were transparent, uniform, and thin (Fig. 3c). *Alternaria alternata* treated with the volatiles, however, did not produce conidia. Due to the low magnification, no differences from controls in the morphology of hyphae were notable under a light microscope (Fig. 3d). SEM revealed that the conidia of normal *A. alternata* in the control group were shaped like an inverted hammer, and mycelia were thin, long, and uniform with smooth surfaces (Fig. 3e). No conidia were observed in the treatment group, and mycelia were fractured, with crumpled surface and damaged morphology (Fig. 3f). A streaking experiment showed that, in the control group, the conidia germinated and grew into colonies (Fig. 3g), but no colonies formed on the plate treated with volatiles (Fig. 3h). This suggests that the volatiles also had an inhibitory effect on conidia germination. In this way, the volatile substances produced by *M. odoratimimus* T45 inhibited *A. alternata*, mainly through suppression of mycelial growth, conidial germination and sporulation, and damage to hyphae morphology.

Antifungal activity of volatiles against eight plant pathogenic fungi

The effect of volatile compounds from *M. odoratimimus* T45 on mycelial growth of eight plant pathogenic fungi (*B. cinerea*, *B. dothidea*, *V. mali*, *C. capsici*, *C. orbiculare*, *G. cingulata*, *F. oxysporum* f. sp. *lycopersici*, *F. oxysporum* f. sp. *vasinfectum*) was investigated. The inhibition rate of mycelial growth of eight plant pathogen ranged from 10.52 to 88.20 % (Table 2). The volatiles significantly inhibited the growth of *V. mali*, *B. dothidea*, and *B. cinerea* with the inhibition rate of 88.20, 86.57, and 80.88 %, respectively. The least pronounced inhibition was observed against *C. orbiculare*, which showed an inhibition rate of only 10.52 %.

Identification of volatile antifungal substances

Volatile compounds produced by T45 and LB liquid medium were analyzed using SPME-GC/MS. The mass spectrum obtained in this way was searched in NIST08 database and compared to standard spectra. A total of 24 compounds identified in the volatiles produced by T45 are shown in Table 3. For the control, 19 compounds were identified (data not shown). These compounds were aldehydes, ketones, hydrocarbons, ethers, alcohols, esters, and acids. Here, 11 compounds were detected in both T45 and the control, in which the concentrations of palmitic acid and stearic acid were both relatively high. For the 13 compounds detected only in the T45 and for palmitic acid and stearic acid, pure commercial compounds

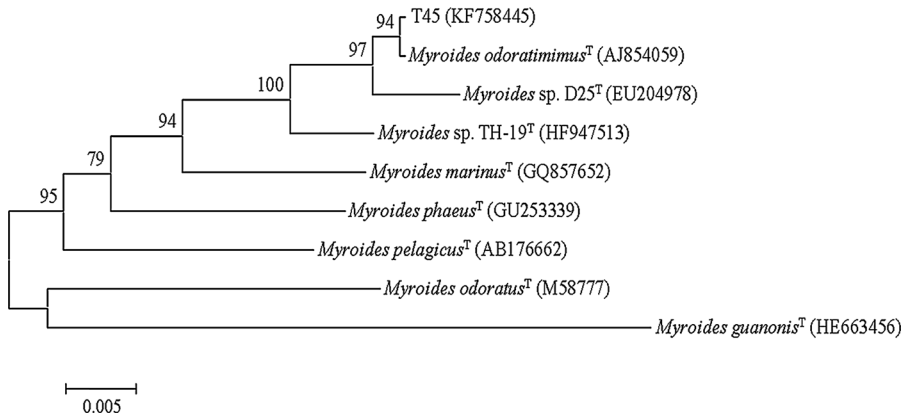

Fig. 2 Molecular phylogenetic tree of bacterium T45 based on 16S rDNA sequence. T45 was identified by 16S rDNA sequence as *Myroides odoratimimus*. The tree was constructed using the neighbor-joining method in MEGA 4.0. Bootstrap values (percent) were determined on the basis of results for 1,000 replicates and are shown for branches with more than 70 % bootstrap support. The scale bar indicates 0.005 nucleotide substitutions per sequence position. Superscripted T indicates sequences of type strains

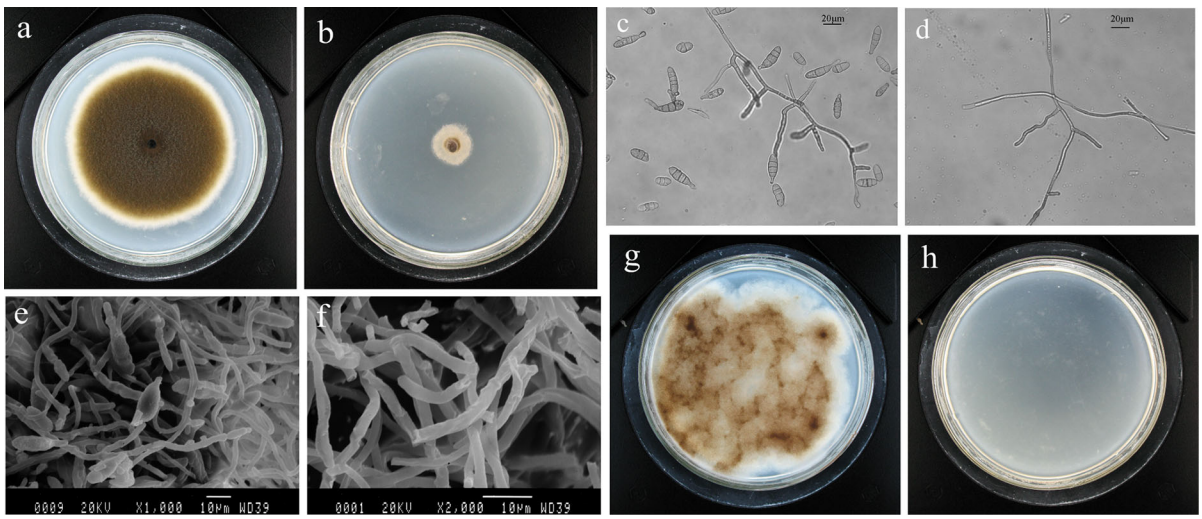

Fig. 3 Inhibitory effect of volatiles from *M. odoratimimus* T45 on *Alternaria alternata*. **a** Normal growth of *A. alternata*. **b** Slow growth of *A. alternata* under the effect of the volatiles. **c** Normal conidia and hyphae. **d** No conidia were observed after treatment with the volatiles. **e** Normal conidia and hyphae under scanning electronic microscopy. **f** No conidia were observed after treatment with the volatiles, hyphae fractured with crumpled surface. **g** Normal conidia germination and colony formation in the control group. **h** Inhibited conidia germination and no colony formation in the treatment group

were used for antifungal activity testing. For five compounds, no pure commercial compounds were available for purchase, leaving a total of ten volatile compounds to be tested for their individual inhibitory effect on *A. alternata*. Among them, seven commercial volatile compounds are available in a liquid state and another three occur in a solid state at normal room temperature. For the seven liquid compounds, SDW was used as control and they were tested at a dose of 100 μl per dish (Table 4). For the solid ones, they were dissolved in methanol at concentration 10 mg ml^{-1} for testing, and methanol was used as control for the three acids (Table 5). Among the ten compounds tested, the inhibitory effects of isoamyl alcohol, isobutyric acid, and isovaleric acid were most prominent, and they completely inhibited the mycelial

Table 2 Antifungal activity of volatiles from *M. odoratimimus* T45 against plant pathogenic fungi

Fungal pathogens	Inhibition rate[1] (%)
Valsa mali	88.20 ± 2.07[a]
Botryosphaeria dothidea	86.57 ± 6.27[a]
Botrytis cinerea	80.88 ± 6.62[a]
Fusarium oxysporum f. sp. *lycopersici*	40.61 ± 3.84[b]
Glomerella cingulata	34.62 ± 3.26[bc]
Colletotrichum capsici	27.55 ± 1.86[c]
Fusarium oxysporum f. sp. *Vasinfectum*	26.34 ± 8.47[c]
Colletotrichum orbiculare	10.52 ± 4.18[d]

Values are the means of three replicates ±SD, inhibition percentage against eight plant pathogenic fungi ($F = 109.77$, $df = 7, 16$, $P < 0.05$). Values within a column followed by the same lower case letters are not significantly different ($P < 0.05$)

[1] Inhibition rate was calculated as percentage mycelial growth inhibition

growth of *A. alternata* at the tested concentration of 100 µl per dish. Benzyl alcohol showed a notable inhibitory effect on *A. alternata* at a dose of 100 µl per dish, but the remaining compounds showed weak or no inhibitory activity.

Discussion

In the present study, bacteria isolated from the tobacco rhizosphere were evaluated for their capacity of producing volatile metabolites inhibiting *A. alternata*. A bacterial strain with high inhibition rate on *A. alternata* was selected, and with 16S rDNA sequencing it was identified as *M. odoratimimus*. Yang et al. (2012) suggested that *M. odoratimimus* 3YW8 isolated from ginger phyllosphere showed antagonism in greenhouse against *Ralstonia* wilt of ginger with the

Table 3 GC–MS analysis of volatile compounds produced by *M. odoratimimus* T45

Possible compound[a]	RT (min)[b]	RA (%)[c]	MW (Da)[d]
Isoamyl alcohol	6.87	3.48	88
Dimethylcyclosiloxane	10.37	12.60	297
Benzaldehyde	11.21	1.19	106
Isobutyric acid	11.92	1.57	88
2(2-Ethoxyethoxy)ethanol	12.42	1.86	134
2-Dodecanone	12.82	1.40	184
Isovaleric acid	13.01	3.85	102
2-Tridecanone	14.62	1.14	198
2-Tetradecanone	15.23	4.13	212
Benzyl alcohol	15.37	3.89	108
3-Tridecanone	15.98	1.54	198
Benzothiazole	16.21	1.61	135
Isopropyl myristate	17.00	2.11	270
Hexadecanol	20.20	7.08	242
δ-Dodecalactone	20.60	1.90	198
Tridecanolactone	21.10	1.54	212
Tridecanoic acid	21.44	1.52	214
Phthalic acid, isobutyl nonyl ester	21.52	1.21	348
1-Nonadecene	21.93	2.19	267
Myristic acid	22.72	1.91	228
Pentadecanoic acid	23.23	1.57	242
Bis(2-ethylhexyl)hexanedioate	24.13	2.20	371
Palmitic acid	25.00	13.37	256
Stearic acid	28.51	3.32	284

[a] The compounds were tentatively identified by mass spectra comparison to those in the NIST 08 Mass Spectral Library (probability based match >80 %)

[b] RT, retention time detected in the GC–MS analysis

[c] RA, relative peak area: the value for a volatile compound represented the percentage of the area of the peak for that volatile compound in the total area of peaks for all the detected volatile compounds

[d] MW, molecular weight

Table 4 Effect of liquid state commercial volatile compounds on the mycelial growth of *A. alternata* taking SDW as control

Volatile compound	Mycelial growth[2] (cm)	Inhibition rate[3] (%)
CK (SDW)[1]	4.52 ± 0.09^{b}	–
Dimethylcyclosiloxane	4.52 ± 0.06^{b}	0^{c}
2-Tridecanone	4.41 ± 0.13^{b}	2.44 ± 2.94^{c}
Benzyl alcohol	1.16 ± 0.24^{c}	74.42 ± 5.24^{b}
δ-Dodecalactone	4.86 ± 0.12^{a}	0^{c}
Isoamyl alcohol	0^{d}	100^{a}
Isobutyric acid	0^{d}	100^{a}
Isovaleric acid	0^{d}	100^{a}

Values are the means of five replicates ±SD, mycelial growth of *A. alternata* ($F = 2{,}160$, $df = 7, 32$, $P < 0.05$), inhibition percentage against *A. alternata* ($F = 2{,}997$, $df = 6, 28$, $P < 0.05$). Values within a column followed by the same lower case letters are not significantly different ($P < 0.05$)

[1] Sterile distilled water was used as control

[2] Mycelial growth of *A. alternata* was determined on potato dextrose agar after incubation at 25 °C for 5–7 days

[3] Inhibition rate was calculated as percentage growth inhibition as compared to SDW

Table 5 Effect of solid state commercial volatile compounds on the mycelial growth of *A. alternata* taking methanol as control

Volatile compound	Mycelial growth[2] (cm)	Inhibition rate[3] (%)
CK (methanol)[1]	3.97 ± 0.05^{ab}	–
Pentadecanoic acid	3.75 ± 0.21^{b}	5.64 ± 5.26^{a}
Palmitic acid	3.90 ± 0.15^{ab}	1.85 ± 3.83^{a}
Stearic acid	4.08 ± 0.05^{a}	0^{a}

Values are the means of five replicates ±SD, mycelial growth of *A. alternata* ($F = 5.34$, $df = 3, 16$, $P < 0.05$), inhibition percentage against *A. alternata* ($F = 3.06$, $df = 2, 12$, $P > 0.05$). Values within a column followed by the same lower case letters are not significantly different ($P < 0.05$)

[1] Methanol was used as control

[2] Mycelial growth of *A. alternata* was determined on potato dextrose agar after incubation at 25 °C for 5–7 days

[3] Inhibition rate was calculated as percentage growth inhibition as compared to methanol

biocontrol efficacy of 59.68 %. The present study is the first report on the antifungal volatile compounds produced by *M. odoratimimus*. Bacteria previously reported to produce volatile substances with antifungal activity mainly include *Streptomyces*, *Pseudomonas*, *Bacillus*, and *Serratia* (Li et al. 2012a).

Volatile metabolites inhibit pathogens mainly via inhibition of mycelial growth, spore germination and spore formation (Fernando et al. 2005; Xu et al. 2004; Li et al. 2010; Arrebola et al. 2010). They also cause abberrance (Arrebola et al. 2010). Studies have shown that different volatiles act on pathogens differently (Liu et al. 2008). Zhou et al. (2011) examined the inhibitory effects of volatile substances produced by a strain of *B. subtilis* on the hyphae of several pathogens. They found that, for *B. dothidea* and *Coniothyrium diplodiella*, the effects were manifested by increased hyphae branching, distortion, and enlarged cells at the tip and in the middle of the hyphae. For *Valsa sordida* and *Helicobasidium purpureum*, the effects were manifested by thickened hyphae, but for *F. oxysporum*, thinned hyphae were observed. Chaurasia et al. (2005) investigated the volatile metabolites of *B. subtilis* and their relationship to the morphology of the hyphae and spores of several fungi. They found that, after treatment, the horizontal and vertical separators in *A. alternata* completely disappeared, and the conidia showed thickened cell walls and were round or irregular in shape. Zhang et al. (2009) reported that the inhibitory effects of volatiles produced by *B. subtilis* strain Tpb55 on *A. alternata* were mainly manifested by inhibition of mycelial growth and

conidia formation. In the present study, the main action mode of volatiles produced by *M. odoratimimus* were found to substantially inhibit *A. alternata* conidia germination, sporulation and mycelial growth and lead to damaged morphology of the hyphae.

SPME-GC/MS analysis revealed that the volatile metabolites of *M. odoratimimus* mainly consisted of alcohols, aldehydes, acids, ketones, esters, and alkanes, consistent with previous reports on the compositions of volatiles produced by bacteria (Fernando et al. 2005; Gu et al. 2007; Wan et al. 2008; Boukaew et al. 2013). Li et al. (2010) examined the effects of the volatile compounds produced by *Streptomyces globisporus* on *Penicillium italicum*. They found that dimethyl disulfide, dimethyl trisulfide, and acetophenone could inhibit mycelial growth, and the inhibition increased as the concentration increased. Fernando et al. (2005) showed that benzothiazole, cyclohexanol, n-decanol, dimethyl trisulfide, ethylhexanol, and nonenal could completely suppress mycelial growth and sclerotia formation of *Sclerotinia sclerotiorum*. Zhang et al. (2010) reported that 5-hydroxy-5-methyl-2-ethylene acid produced by *Actinoplanes* sp. exhibited strong antifungal activity against *B. cinerea*, *Cladosporium cucumerinum*, and *Corynespora cassiicola*. The antifungal activities of volatile compounds typically show the following pattern: acids > aldehydes > alcohols > ethers > ketone > ester > lactone (Maruzzella et al. 1961; Hitoshi et al. 2012). In the current experiment, a two-compartment plate method was used to test the antifungal activity of individual compounds. Benzyl alcohol showed a substantial inhibitory effect on *A. alternata*, but isoamyl alcohol, isobutyric acid, and isovaleric acid were found to completely inhibit the growth of the pathogen at a dose of 100 µl per dish. Hitoshi et al. (2012) reported that isoamyl alcohol produced by yeast *Candida maltosa* NP9 could inhibit the germination of fungal spores. Previous studies have shown that isobutyric acid is the main component of volatile compounds produced by endophytes and is most likely to have antifungal activity (Corcuff et al. 2011). The inhibitory effect of isovaleric acid on the growth of pathogenic fungi has not yet been reported. The use of the three antifungal volatiles of *A. alternata* observed in biocontrol requires further study.

Ryu et al. (2003) first reported that bacterial volatiles dibutoxide and acetyl methyl carbinol could promote the growth of *Arabidopsis*. Recent studies have shown that volatile metabolites of rhizosphere bacteria that can promote plant growth are ubiquitous (Gutiérrez-Luna et al. 2010). Farag et al. (2006) showed that many branched alcohols (mainly isoamyl alcohol, 2-methyl-1-butanol, and their conjugates) were also likely to be growth-promoting. Hung et al. (2013) showed that volatiles produced by *Trichoderma viride* could significantly promote *Arabidopsis* growth. In that study, isoamyl alcohol was one of the volatile components with the highest concentrations. In the present study, the active ingredients of antifungal volatile compounds produced by *M. odoratimimus* were found to include isoamyl alcohol, isobutyric acid, and isovaleric acid. Their ability to promote plant growth needs to be explored in further studies.

In conclusion, volatiles produced by *M. odoratimimus* T45 suppressed the growth of *A. alternata* and showed different degrees of inhibitory effect on eight common plant pathogen effectively. *M. odoratimimus* T45 and volatiles it produced, especially isoamyl alcohol, isobutyric acid, and isovaleric acid, could be considered biocontrol agents. Fumigation experiments will be discussed in the next step.

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